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ORIGINAL ARTICLE

Single closed tube Tm-shift method versus PCR-Restriction Fragment Length Polymorphism in detection of Macrophage Migration Inhibitory Factor-173 G>C (*s755622*) single nucleotid polymorphism in patients with rheumatoid arthritis

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ABSTRACT

Background: *Macrophage migration inhibitory factor (MIF) might has a role in the development and disease activity of Rheumatoid arthritis (RA). There are several disadvantages in the conventional methods for MIF genotyping and there is a strong need for convenient, flexible, accurate and inexpensive method.* ***Objectives:*** *This study was designed to study the application of single closed tube melting temperature (Tm)-shift method in genotyping of MIF-173 G>C (s755622) gene in RA patients and to compare its result with the conventional PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) and to investigate the potential association of these variants of MIF gene with susceptibility and activity of RA.* ***Methodology:*** *GC-rich tail was attached to 5'-end of one of the two allele specific forward primers, such that MIF alleles can be discriminated by the Tm of the PCR products. One hundred RA patients and 40 healthy controls were genotyped for MIF-173 G>C by both Tm-shift method and conventional PCR-RFLP.* ***Results:*** *The comparison of the PCR-RFLP and the Tm-shift method showed one discordant result in 140 samples tested. Retesting this sample with prolonged time of incubation with the restriction enzyme, corrected the result to become 100% agreement between the two methods. No significant association could be found between MIF-173 G>C SNP and risk of RA. C/C genotype is more prone to higher disease activity than other genotypes.* ***Conclusion:*** *The single closed-tube Tm-shift method is reliable, rapid and cost-effective and it is superior to conventional PCR-RFLP in genotyping of MIF.*

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune chronic inflammatory disease that affects the synovial joints causing synovitis and progressive cartilage erosions and destruction. RA affects ≈1% of the population, and it predominates in females. Although the full etiology of RA is not clear, it is suggested that a combination of environmental and genetic factors contributes to the autoimmune manifestations of RA [1].

Macrophage migration inhibitory factor (MIF) is a lymphokine that inhibits random migration of macrophages and recruits them at inflammatory loci. MIF is recognized to be a key regulator of the adaptive and innate immune responses; it stimulates the expression and secretion of pro-inflammatory mediators such as: TNF-α, IL-1β, IL-2, IL-6, IL-8 and IL-12 [2]. Also, MIF acts by inhibition of p53 dependent apoptosis, and potentiates activation and proliferation of T cells. MIF also has counter-regulatory effect on the immunosuppressive action of glucocorticoids, and therefore has a role in determining the magnitude of the inflammatory response [3].

A 'G' to 'C' single nucleotide polymorphism (SNP) at the -173 position in MIF gene has been described. This polymorphism creates a site for binding of activator protein-4 (AP-4) transcription factor and is associated with increased MIF gene expression and protein levels and hence, increased susceptibility to different inflammatory and autoimmune diseases [4].

Genetic polymorphisms change the structure and quantity of the gene product; and accordingly the function of the product, and hence the genetic differences between individuals are largely associated with disease susceptibility and determine response to therapy [5].

Despite many advances in technologies of SNP genotyping, there is no clearly superior method has been established and there is still a need for convenient, flexible and inexpensive methods with a reasonable throughput that can be quickly adopted in various laboratories [6].

The majority of genotyping methods such as hybridizations, digestion with restriction enzyme, physical separations, and sequencing rely on PCR amplification, followed by additional identification and resolution steps. Techniques combining amplification and detection have been developed but require either expensive probes or individual reactions for each allele. These methods have different requirements, advantages, and limitations [7].

A single closed-tube melting temperature (Tm)-shift genotyping method has been developed. The goal of this method was to combine several techniques for detecting both alleles in one reaction and, in real time, by use of a standard dye SYBR Green with no need for additional steps [6].

The principle of this method rely on fluorescence melting curve analysis that can provide single-tube amplification and detection of polymorphic alleles, if the melting temperatures of the two alleles are sufficiently different [8]. This can be achieved by introduction of a 10- to 15-bp GC clamp onto the 5′end of one of the two allele specific forward primers, thus, generating two allele-specific PCR amplicons with different sizes. As the amplicon melting temperature depends on its size, the two different sizes of allele-specific amplicons result in two different melting temperatures in dissociation curve analysis permitting complete discrimination between the two polymorphic alleles in the same well [6].

This study was designed to explore the application of single closed tube Tm-shift method in genotyping of MIF-173 G>C (s755622) gene and to compare its result with the standard PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) and to investigate the potential association of these MIF genetic variants with RA susceptibility and activity.

METHODOLOGY

This study was carried out on 100 patients diagnosed RA, attending the Out-Patient Clinic and inpatient of Rheumatology and Rehabilitation Department, Benha University hospital during the period from October 2017 to October 2018. The RA patients were diagnosed according to the American Rheumatism Association 1987 revised criteria [9]. As a control group, 40 unrelated, healthy subjects matched in age and sex with RA patients were included. Disease activity was evaluated in the RA group by means of 28-joint disease activity score (DAS28 index) [10].

Each participant was subjected to genotyping of MIF-173 G>C (s755622) gene by two PCR methods: PCR–RFLP [11] and Single-tube PCR with Tm-shift primers [6].

This work was approved by the Ethics Committee for Human Research of Benha University. Informed consent was taken from all participants.

Genomic DNA extraction

Genomic DNA was extracted from 5 ml of whole blood collected on tubes containing EDTA by Whole Blood Genomic DNA Purification Mini Kit (ThermoScientific, Lithuania) according to the manufacturer's instructions. The yields of DNA were measured from the DNA concentration in the elute measured by absorbance at 260 nm. Also, the A260/A280 ratio was calculated to ensure the purity of the DNA (ratio between 1.8-2.0). The extracted DNA was then stored at -20°C until further processing.

Genotyping of MIF-173 G>C (s755622) gene using PCR–RFLP

Primers were designed for amplification of MIF-173 G>C gene to be suitable for PCR for this gene [11]. The sequence of forward primer was: 5'-ACT-AAG-AAA-GAC CCG-AGG-C-3', and that of reverse primer was: 5'-GGG-GCA-CGT-TGG-TGT-TTA-C-3'. (Dream Taq green PCR master Mix 2x) supplied by (Fermentas, Germany) was used for amplification. 50 ul reaction contained 25 ul of Taq PCR master Mix 2x, 3 ul of each of the two primers, 6 ul of the template DNA and 13 ul of nuclease free water. For amplification, ***G*** storm thermal cycler UK was used with the following conditions : initial denaturation at 95 ºC for 2 min. ,40 cycles of denaturation at 95 ºC for 30 s, annealing at 60 ºC for 30 s and extension at 72 ºC for 45 s , followed by final extension at 72 ºC for 10 min. The amplified 366 pb PCR products were digested by restriction enzyme *AluI* (ThermoScientific, Lithuania) as follow: Preparation of a mixture of 10 ul of PCR reaction mixture, 18 ul of nuclease free water, 2 ul of 10X Buffer Tango and 2 ul of *AluI*. The product was mixed gently and spinned down for few seconds, incubated at 37 for 4 hours. Then thermal inactivation was done at 65 ºC for 20 min .This protocol was done in Thermo cycler (Hybaid, USA). The restriction fragments were identified with 2% agarose gel electrophoresis.

Genotyping of MIF-173 G>C (s755622) gene using Single-tube PCR with Tm-shift primers

For analysis of allelic variants, two allele-specific forward primers were designed, with the 3′ base of each primer matching only one of the biallelic SNP bases to be evaluated. A common reverse primer was designed downstream of the polymorphic site. A GC-rich tail was added to the 5′ end of one allele-specific primer to differentiate PCR products on the basis of their melting temperatures and to achieve a difference of 4°C or more between the two alleles. For more robust assay, additional mismatches were added near the 3′ end of specific primers to avoid formation of primer–dimers or non-specific amplicons. The primer sequences [12] are illustrated in table 1.

A7900 HT Fast Real-Time PCR System instrument (Applied Biosystems, Foster City, CA, USA) was used for PCR amplification and fluorescence melting curve analysis. PCR was performed with volume of 25 µL

reactions contained: 12.5 µl of Maxima SYBR Green qPCR Master Mix (2X) (ThermoScientific, Lithuania), 0.05 µl ROX solution, 2.2 µl of each of the three primers, 1.9 µl of nuclease-free water and 3.95 µl of template DNA. Amplification conditions consisted of initial activation of the *Taq* DNA polymerase by incubation at 95°C for 10 min., then 40 cycles denaturation for 20 s at 95°C, followed by annealing for 30 s at 60°C and extension at 72°C for 1 min. Then, the intensity of the fluorescence of the PCR product was measured from 60°C to 95°C at a temperature gradient of 0.2°C/min. The 7900 HT Fast Real-Time PCR System automatically calculates the negative derivative of the change in fluorescence. When graphed, this yields a peak at the Tm of the PCR product.

Table 1: Primer sequences for the genotyping of MIF-173 G>C by Tm shift method.

	Primer sequence (5'-3')
F-wt	CGC CAA GTG GAG AAC AcG**G**
F-mt	GCGCGCGCGCGCGCG CGC CAA GTG GAG AAC AaG**C**
R-com	GCA GAG GCA CAG ACG CA

(The polymorphic base is in bold, the 3′ mismatch is in lowercase and the GC clamp is underlined).

Statistical Analysis

The collected data were tabulated and analyzed using SPSS version 16 soft ware (SpssInc, Chicago, ILL Company. Categorical data were presented as number and percentages. Z test of proportions ($Z_{Prop.}$) and Fisher's exact test (FET) were used to analyze them. Cohen Kapaa test (k) was used to assess degree of agreement between the techniques. Quantitative data were tested for normality using Kolomogrov Smirnove test, assuming normality at P>0.05. Non parametric variables were presented as median and range and analyzed using Kruskal Wallis test (KW) for difference among 3 independent groups. P≤ 0.05 was considered significant. Genotype distributions in the studied groups were in Hardy-Weinberg equilibrium for gene polymorphisms (data not shown). Hardy-Weinberg equilibrium was calculated according to OEGE - Online Encyclopedia for Genetic Epidemiology studies [13].

RESULTS

The RA patients were 27 males and 73 females with median age (range) of 52, (33-75). Mean DAS28 was 4.58 ± 1.47 with 24% of patients were in disease remission (DAS28 ≤ 2.6) and 76% are in active form of the disease (DAS28 > 2.6).

The results of genotyping of polymorphic restriction sites in the MIF gene are shown in Figure 1. MIF genotypes were determined by the patterns of these fragments from gel documentation system obtained from PCR-RFLP as follows: the wild-type homozygous (G/G): 268 bp and 98 bp, mutant homozygous (C/C): 205 bp, 98 bp and 63 bp while mutant heterozygous (C/G): 268 bp, 205 bp, 98 bp and 63 bp.

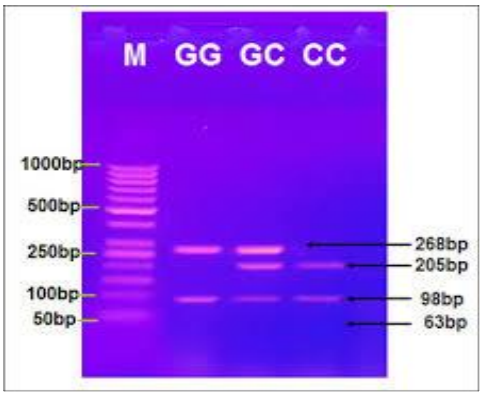

Fig. 1: MIF-173 G>C genotyping by PCR-RFLP. M: DNA ladder (50-1000 bp), G/G (wild-type homozygous): 268 bp and 98 bp, C/G (mutant heterozygous): 268 bp, 205 bp, 98 bp and 63 bp, C/C (mutant homozygous): 205 bp, 98 bp and 63 bp.

Dissociation curve for MIF-173 G>C SNP is shown in Figure 2. The wild-type homozygous (G/G) peaked at 79.38°C ± 0.30°C, whilst mutant homozygous (C/C) produced a single peak at 84.83°C ± 0.23°C. Both peaks were present in heterozygous individuals (C/G). The difference of Tm between G and C allele is approximately 5.5 °C, which enables sufficient discrimination between them in melting curves.

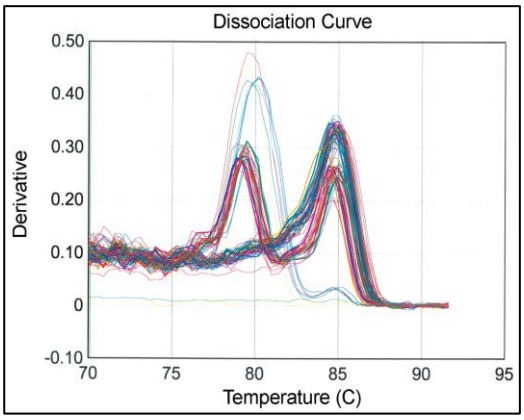

Fig. 2: MIF-173 G>C Tm curves. wild-type homozygous (G/G) peaked at 79.38°C ± 0.30°C. Mutant homozygous (C/C) peaked at 84.83°C ± 0.23°C. Heterozygous (C/G) includes both peakes.

Genotype and allele frequencies of MIF-173 G>C in RA patients and controls are illustrated in Table 2. There were no significant differences in genotypes and alleles frequencies between RA patients and controls and hence, no significant association could be found between MIF-173 G>C SNP and risk of RA.

Table 2: Alleles and genotypes frequencies distribution of MIF-173 G>C SNP in RA patients and controls

	RA patients (n=100)		Controls (n=40)		$Z_{Prop.}$	P value	OR (95% CI)
	N	%	n	%			
G/G	63	63	26	65	Reference category		
C/G	28	28	10	25	0.33	0.74 (NS)	1.15 (0.49-2.7)
C/C	9	9	4	10	0.12	0.9 (NS)	0.92 (0.26-3.3)
Allele G	154/200	77.0	62/80	77.5	Reference category		
Allele C	46/200	23.0	18/80	22.5	0.091	0.92 (NS)	1.03 (0.55-1.9)

OR: Odds ratio. 95% CI: 95% Confidence Interval. NS: Non significant .

As regard disease activity, the median DAS28 score is significantly higher in C/C than other genotypes. Also, there is significant increased frequency of CC genotype in patients with the high activity (> 5.1) of RA (Table 3, Figure 3).

Table 3: Disease activity index (DAS28) among different MIF-173 G>C genotypes in RA patients

	G/G n = 63	C/G n = 28	C/C n = 9	Test of significance	P
DAS28 (median- range)	3.3 (2-6.7)	3.05 (2-7.3)	6.1*† (3.5-8.9)	KW test=14.4	=0.001 (HS)
DAS28 ≤ 2.6 (In remission) (n = 24) n (%)	17(27.0)	7 (25.0)	0 (0.0)	FET=16.4	0.006 (S)
3.2 ≤DAS28 > 2.6 (Low activity) (n = 23) n (%)	14 (22.2)	9 (32.1)	0 (0.0)		
5.1 ≤ DAS28 >3.2 (Moderate activity) (n = 33) n (%)	23 (36.5)	8 (28.6)	2 (22.2)		
DAS28 >5.1 (High activity) (n = 20) n (%)	9 (14.3)	4 (14.3)	7 (77.8)		

*→ significant in comparison with GG, †→ significant in comparison with CG

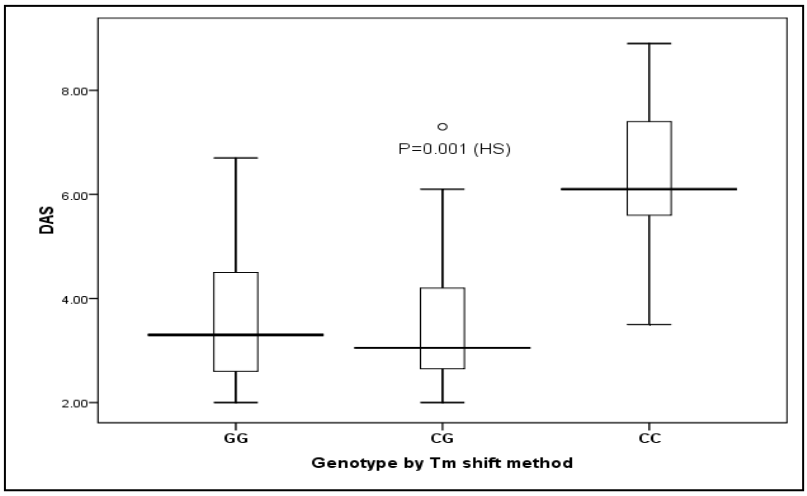

Fig. 3: Box plot showing median and range of DAS28 according to genotype.

The comparison between the two PCR methods showed one discordant result in patients samples. By initial PCR-RFLP testing, this sample was typed as G/G, whereas this sample was typed as C/C by initial Tm-shift method (Degree of agreement 99%). This sample was retested by the two methods. In Tm-shift method, the result was confirmed as C/C. In PCR-RFLP, the PCR products were re-incubated for prolonged time (overnight) with the restriction enzyme *AluI*, and then the result was changed from G/G to C/C, and thus the degree of agreement become 100% between the two methods (Table 4,5).

Table 4: Degree of agreement between PCR-RFLP (after 4 hours incubation with *AluI*) and Tm shift method.

Genotype by			Tm shift method			Total
			G/G	C/G	C/C	
PCR-RFLP	G/G	Count	63	0	1	64
		% within genotype by Tm shift method	100.0%	.0%	11.1%	64.0%
	C/G	Count	0	28	0	28
		% within genotype by Tm shift method	.0%	100.0%	.0%	28.0%
	C/C	Count	0	0	8	8
		% within genotype by Tm shift method	.0%	.0%	88.9%	8.0%
Total		Count	63	28	9	100
		% within genotype by Tm shift method	100.0%	100.0%	100.0%	100.0%

Kappa test=0.980 P<0.001 (HS) Degree of agreement = 99%

Table 5: Degree of agreement between PCR-RFLP (after overnight incubation with *AluI*) and Tm shift method.

Genotype by			Tm shift method			Total
			G/G	C/G	C/C	
PCR-RFLP	G/G	Count	63	0	0	63
		% within genotype by Tm shift method	100.0%	.0%	.0%	63.0%
	C/G	Count	0	28	0	28
		% within genotype by Tm shift method	.0%	100.0%	.0%	28.0%
	C/C	Count	0	0	9	9
		% within genotype by Tm shift method	.0%	.0%	100.0%	9.0%
Total		Count	63	28	9	100
		% within genotype by Tm shift method	100.0%	100.0%	100.0%	100.0%

Kappa test=1.0 P<0.001 (HS) Degree of agreement = 100%

DISCUSSION

Autoimmune disorders are complex pathologies that involve both environmental and genetic factors. The investigation of gene polymorphisms involved in the pathogenesis of this sort of diseases is a very important concern [2]. Several studies have reported an association between MIF-173 G>C gene and RA susceptibility [1,14,15,16]. However, in contrast, other studies reported that MIF-173 G>C is not associated with RA susceptibility [4,17,18], which come in agreement with our result. These different conclusions regarding susceptibility may be related to genetic differences in the populations studied or due to differences in the sample sizes.

The association of MIF-173 G>C gene with disease activity also was investigated. We detected that carriers of the C/C genotype were more prone to higher disease activity than carriers of the other genotypes. This comes in agreement with previous reports [4,19].

This work studied the application of single closed tube Tm-shift method for genotyping of MIF-173 G>C SNP and compared its results with that of the standard PCR-RFLP. The result showed one discordant result between the two genotyping methods (one sample was determined as G/G with PCR-RFLP analysis while determined as C/C by single-tube with Tm shift primers procedure). This difference observed was explained by incomplete digestion of the amplicons in PCR-RFLP protocol, as when the PCR products of this sample was re-incubated for prolonged time (overnight incubation) with the restriction enzyme, the result changed to become C/C and the result became 100% agreement between the two methods.

This result comes in agreement with a study [20] whose authors reported that, because incomplete digestion of the restriction enzyme can lead to false genotyping data, many researchers prefering the overnight digestions, and this will increase the assay time even further. Also,

another study [21] reported that PCR-RFLP is error-prone method due to possible incomplete digestion of the restriction enzyme.

Several works studied and evaluated single closed tube Tm-shift technique for detection of different gene polymorphism [22,23,24]. In a work [23] that studied this technique for genotyping of human platelet antigen-15 (HPA-15) in 100 samples of blood and compared its results with that of conventional polymerase chain reaction with sequence-specific primers (PCR-SSP), the results showed four discordant results. Confirmatory results found that the PCR-SSP showed several errors, whereas the results of retesting of Tm-shift method were identical with the results of the initial testing.

Also, another work[24] compared single closed tube Tm-shift method with PCR-based Sanger sequencing in genotyping of two single nucleotide polymorphisms of APOE gene, rs429358 and rs7412, in 300 subjects. A 100% agreement was obtained between the two approaches.

General criteria for selecting SNP genotyping methods include accuracy/sensitivity, success rate, flexibility, rapidity, cost and throughput [6]. A major advantage of single-tube PCR with Tm-shift primers approach observed in this study is rapidity, the genotyping of the samples was done and completed within three hours. In contrast, conventional PCR-RFLP protocol was time consuming, it took at least ten hours, and in case of overnight incubation with the restriction enzyme, it took more than 24 hours. Many researchers [6,7] concluded that, although PCR-RFLP considered the standard genotyping technique, it is time consuming especially when large series of samples and if there is more than one nucleotide variation have to be analyzed, due to several steps that needed to be sequentially performed, including amplification, restriction enzyme digestion, gel electrophoresis and documentation of the electrophoretically separated DNA fragments. So, this method is not suitable for testing a big number of different SNPs at the same time.

As regard the cost, *Calero et al, (2009)* [25] in their studies, reported that, the main advantage of single-tube PCR with Tm-shift primers is its reduced cost and relative simplicity compared to other techniques, such as the FRET and TaqMan assays. This reduced cost is mainly related to the use of non-labelled primers, which are standard DNA oligonucleotides, instead of expensive labelled probes.

Indeed, Tm-shift genotyping comes with few material requirements. Once allele-specific primers have been designed for a particular SNP, a big number of individual samples can be processed with use of the generic SYBR Green fluorescent dye only. So, this technique allows a high-throughput typing of significant SNPs, both for medical diagnostics and for gene mapping [6].

In contrast, PCR-RFLP needs many equipment beyond PCR thermal cycler such as thermal block, gel electrophoresis unit, UV transilluminator and gel documentation system. Moreover, it needs a restriction enzyme or more (in the event that there are two or more nucleotide variation in the restriction enzyme recognition site), reagents used for gel preparation and PCR marker. So, the cost of this technique was considered high compared to the GC clamp allele-specific assay as a real time technique [26].

In single-tube PCR with Tm-shift primers approach, neither post PCR restriction enzyme digestion nor gel electrophoresis be required. This technique has an advantage of that all reactions and measurements take place in a single closed tube, eliminating manipulation of PCR product and removing the risk of post- PCR contamination. Moreover, this technique offers the practice of a very elegant approach not prone to mistakes as it requires only minimal human intervention unlike PCR-RFLP which needed several manual sample-processing steps [22].

The process of single-tube PCR with Tm-shift uses end point analysis of PCR product so that real time monitoring of PCR progression or cycle threshold is not necessary. Performing PCR in separate thermal cyclers and then evaluating the fluorescence melting curve of the product in a dedicated instrument could further increase throughput. This method is suitable for the medium to high-volume laboratory which desire inexpensive technique for rapid DNA polymorphism analysis [7].

CONCLUSION

In summary, the single-tube PCR with Tm-shift assay described here meets all the criteria required by a laboratory involved in complex diseases studies. It is fast, simple, inexpensive, reliable and has a high throughput and flexibility to suit different sample size that can be used efficiently in different research and clinical laboratories.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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